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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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INVENTOR(S)		
Given Name (first and middle (if any))	Family Name or Surname	Residence (City and either State or Foreign Country)
Dominique P.	BRIDON	Town of Mont-Royal, Quebec, CANADA
Jean-Paul	CASTAIGNE	Town of Mont-Royal, Quebec, CANADA
Xicai	HUANG	Kirkland, Quebec, CANADA

[X] Additional Inventors are being named on the 1 separately numbered sheets attached hereto

TITLE OF THE INVENTION (500 characters max)

LONG LASTING INSULIN DERIVATIVES AND RELATED METHODS

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[X] Customer Number [ ] → Customer Number

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[ ] Firm or Individual Name					
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City		State		Zip	
Country		Telephone		Fax	

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- [X] Applicant claims small entity status. See 37 CFR 1.27.
- [ ] A check or money order is enclosed to cover the filing fees
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Respectfully submitted,

SIGNATURE

TYPED OR PRINTED NAME Timothy A. Worrall

TELEPHONE (415) 268-7151

Date July 25, 2003

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USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

I hereby certify that this correspondence is being deposited with the U.S. Postal Service as Express Mail, Airbill No. EV 302 339 381 US in an envelope addressed to: Box Provisional Patent Application, Commissioner for Patents, Alexandria, VA 22313-1450, on the date shown below.

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PROVISIONAL APPLICATION COVER SHEET
Additional Page

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INVENTOR(S)/APPLICANT(S)

Given Name (first and middle (if any))	Family or Surname	Residence (City and either State or Foreign Country)
Roger Martin	LEGER ROBITAILLE	Saint-Lambert, Quebec, CANADA Granby, Quebec, CANADA

[redacted]

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Applicant Information	
Applicant Authority Type:	**Inventor**
Primary Citizenship Country::	UNKNOWN
Status::	Full Capacity
Given Name::	**DOMINIQUE**
Middle Name::	**P.**
Family Name::	**BRIDON**
City of Residence::	Town of Mont-Royal, Quebec
Country of Residence::	CANADA
Street of mailing address::	1375 Scarboro Road
City of mailing address::	Town of Mont-Royal, Quebec
Country of mailing address::	CANADA
Postal or Zip Code of mailing address::	H3P 2S2
Applicant Authority Type:	**Inventor**
Primary Citizenship Country::	UNKNOWN
Status::	Full Capacity
Given Name::	**JEAN-PAUL**
Middle Name::	
Family Name::	**CASTAIGNE**
City of Residence::	Town of Mont-Royal, Quebec
Country of Residence::	CANADA
Street of mailing address::	455 Lockhart Avenue
City of mailing address::	Town of Mont-Royal, Quebec
Country of mailing address::	CANADA
Postal or Zip Code of mailing address::	H3P 1Y6

[redacted]

Applicant Authority Type::	**Inventor**
Primary Citizenship Country::	UNKNOWN
Status::	Full Capacity
Given Name::	**XICAI**
Middle Name::	
Family Name::	**HUANG**
City of Residence::	Kirkland, Quebec
Country of Residence::	CANADA
Street of mailing address::	153 Denault
City of mailing address::	Kirkland, Quebec
Country of mailing address::	CANADA
Postal or Zip Code of mailing address::	H9J 3X2

Applicant Authority Type:	**Inventor**
Primary Citizenship Country::	UNKNOWN
Status::	Full Capacity
Given Name::	**ROGER**
Middle Name::	
Family Name::	**LEGER**
City of Residence::	Saint-Lambert, Quebec
Country of Residence::	CANADA
Street of mailing address::	202 Upper Edison
City of mailing address::	Saint-Lambert, Quebec
Country of mailing address::	CANADA
Postal or Zip Code of mailing address::	J4R 2V8

Applicant Authority Type:	**Inventor**
Primary Citizenship Country::	UNKNOWN
Status::	Full Capacity
Given Name::	**MARTIN**
Middle Name::	
Family Name::	**ROBITAILLE**
City of Residence::	Granby, Quebec
Country of Residence::	CANADA
Street of mailing address::	491 Frechette
City of mailing address::	Granby, Quebec
Country of mailing address::	CANADA
Postal or Zip Code of mailing address::	J2G 6A2

Correspondence Information

Correspondence Customer Number::	20872

Representative Information

Representative Customer Number::	20872

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Long Lasting Insulin Derivatives And Related Methods

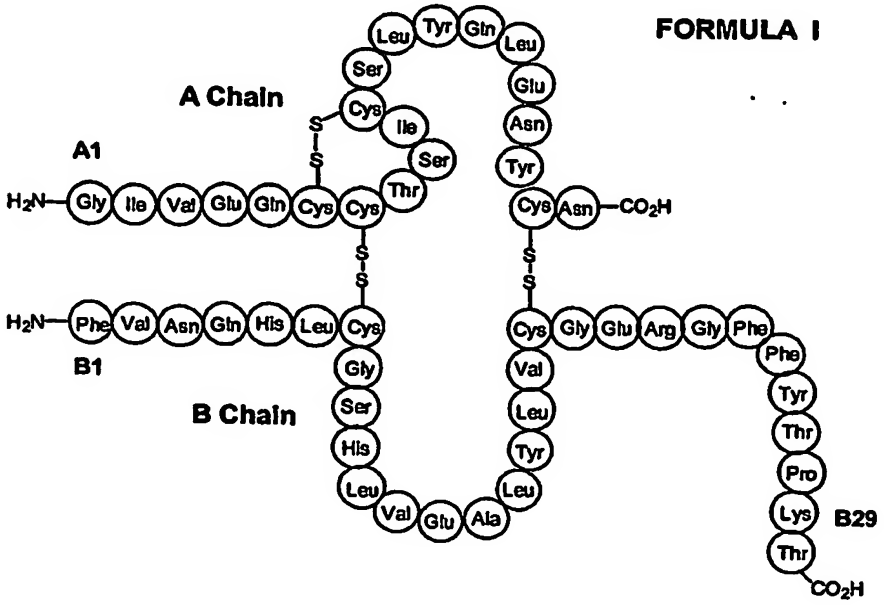
DESCRIPTION OF THE INVENTION :

The invention relates to a long lasting insulin derivative. More particularly, the insulin derivative comprises an insulin molecule and a reactive group capable to covalently bond a blood component *in vivo* or *ex vivo*.

The insulin molecule may be native human insulin or an analogue thereof such as an insulin molecule with amino acid substitution(s), amino acid deletion(s) or amino acid addition(s). An example of an insulin analogue is insulin glargine provided by Lantus®. Another example of an insulin analogue is native human insulin the amino acid in position B30 deleted.

The reactive group may be coupled to different functionalities on the insulin molecule or analogue thereof. According to preferred embodiments of the invention, the reactive group is coupled to available amino groups of the insulin molecule. The molecule of native human insulin is illustrated in formula I:

FORMULA I



According to said preferred embodiments of the invention, the reactive group is coupled to an amino acid selected from the ones in positions A1, B1 and B29 i.e. the α-amino group of the amino terminal amino acid of chains A and B, and the ϵ-amino group of the lysine residue in position 29 of chain B. In accordance to the invention, analogue of insulin containing substituted and/or added amino acid(s) may contain additional amino group for coupling the reactive group; or other functionalities appropriate for coupling the reactive group.

Preferred reactive groups capable to covalently bond a blood component *in vivo* or *ex vivo*, are succinimidyl-containing groups and maleimido-containing groups. The more preferred reactive group is a maleimido-containing group, and more particularly MPA.

Optionally, the reactive group can be coupled to the insulin molecule via a linker. The linker is preferably AEEA, AEEA-AEEA, EDA or $-NH_2-(CH_2)_n-COOH$ where n is an integer between 1 and 20. The more preferred linker is $-NH_2-(CH_2)_7-COOH$, also named 8-amino caprylic acid.

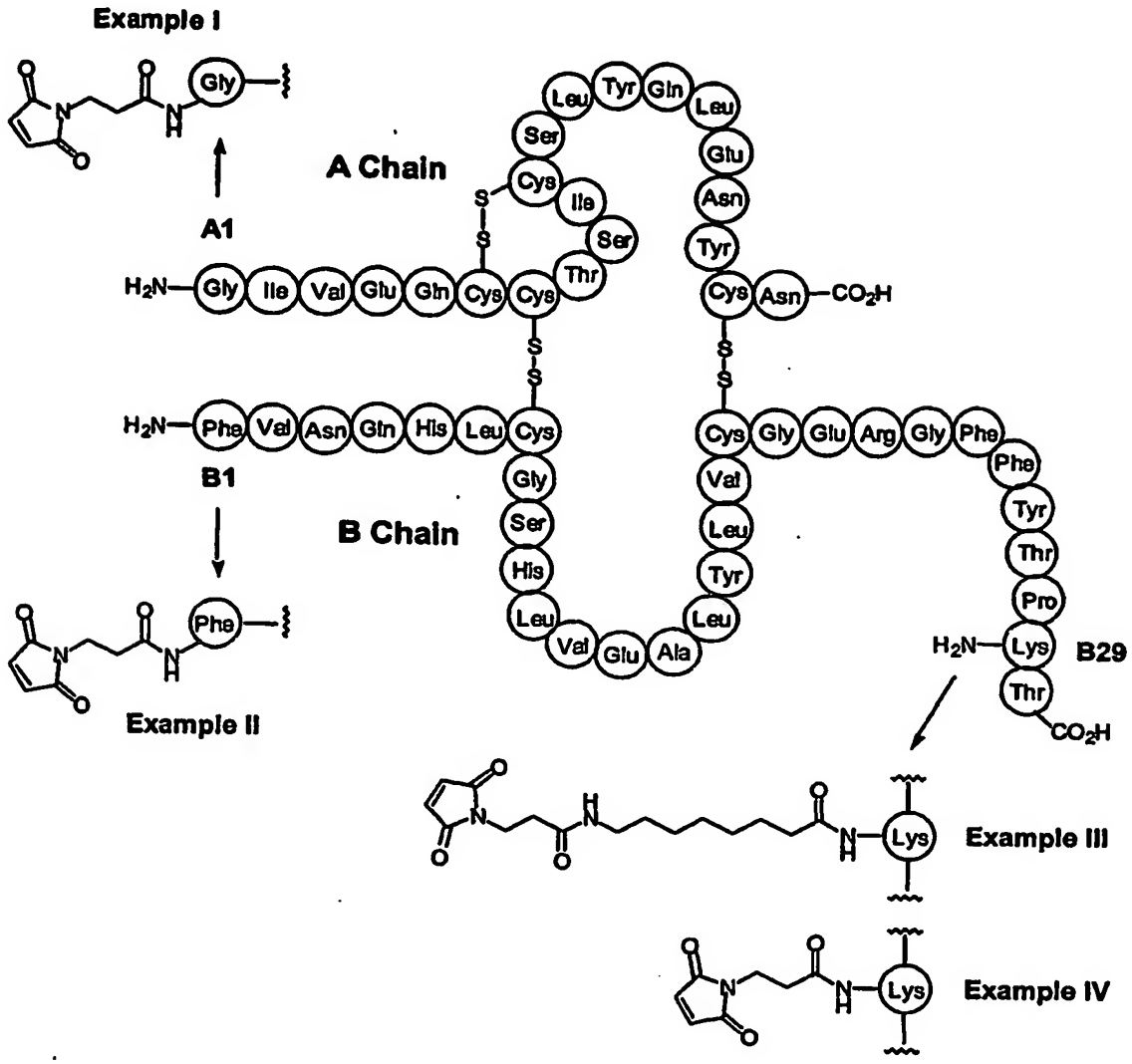
The present invention also relates to an insulin conjugate. The conjugate comprises an insulin derivative where its reactive group has reacted with a blood component *in vivo* or *ex vivo* so as to form a covalent bond. Therefore, the conjugate may be formed *in vivo* by the administration of the insulin derivative, or *ex vivo* by contacting the insulin derivative and blood or purified blood components *in vitro* and allowing formation of the covalent bond. Purified blood components can be provided by purification of a type of blood component from blood sample or produced by recombinant techniques. The preferred blood component is a blood protein, and more preferably, serum albumin.

The present invention further relates to method for treating glycaemic-related diseases or disorders, comprising the administration of insulin derivatives or insulin conjugates being prepared *ex vivo*. Of course, glycaemic-related diseases or disorders include diabetes of Type I and II, and gestational diabetes. Also, cystic fibrosis, polycystite ovary syndrome, pacreatitis and other pancreas-related diseases may also be treated by the administration of insulin derivatives or insulin conjugates according to the present invention. Insulin is also known as a growth factor and is therefore useful in topical administration for wound healing and other related applications.

EXAMPLES:

Synthesis of Insulin Derivatives:

FIGURE 1



Example I

Synthesis of (A1)-MPA-Insulin. Insulin (100 mg) was dissolved in DMF (2 mL) and TFA (100 uL). To the solution, NMM (4-methylmorpholine, 200 uL) and MPA-OSu (N-succinimidyl 3-maleimidopropanoate, 9.2 mg 2.5 equivalents) were added and the reaction was stirred for 2 h. The reaction was quenched by addition of water and adjusted to pH 4 with AcOH. Acetonitrile was added to dissolve the precipitate and the total volume of water/acetonitrile (3:1) was 20 mL. The solution was injected into a semi-preparative HPLC. Phenomenex Luna 10 m phenyl-hexyl 21 mm X 250 mm column equilibrated with an aqueous TFA solution (0.1% TFA in H_2O, solvent A) and an acetonitrile TFA solution (0.1% TFA in acetonitrile, solvent B). Elution was achieved at 9.5 mL/min by running a 27 to 31% B gradient over 120 min. Fractions containing peptides were detected by UV absorbance at 214 and 254 nm. Fractions were collected in 9.5 mL aliquots. Fractions containing the desired product profile were identified by mass detection after direct injection onto LC/MS. The pure fractions at Rt= 36-46 min. were collected, combined and lyophilized to give a white powder (40 mg) along with 23 mg of recovered insulin.

Mass calculated is 5958.5 g/mol, and measured by LC-MS is 5958.0 g/mol.

The amino acid sequence analysis (Edman degradation using phenylisothiocyanate) was used to confirm that the A-chain N-terminal was blocked and B-chain N-terminal (phenylalanine) was still free.

Example II

Synthesis of (B1)-MPA-Insulin. Insulin (100 mg) was dissolved in DMSO (4 mL) and Et_3N (100 uL) with sonication. To the solution, Boc_2O (9.3 mg, 2.5 equivalents) was added and the reaction was stirred at ambient temperature for 30 min. The reaction was quenched by addition of water (15 mL) and acetonitrile (5 mL) and the solution was adjusted to pH 4 with AcOH. The solution was injected into a semi-preparative HPLC. Phenomenex Luna 10 m phenyl-hexyl 21 mm X 250 mm column equilibrated with an aqueous TFA solution (0.1% TFA in H_2O, solvent A) and an acetonitrile TFA solution (0.1% TFA in acetonitrile, solvent B). Elution was achieved at 9.5 mL/min by running a 27 to 40% B gradient over 120 min. Fractions containing peptides were detected by UV absorbance at 214 and 254 nm. Fractions were collected in 9.5 mL aliquots. Fractions containing the desired product profile were identified by mass detection after direct

injection onto LC/MS. Three products (Boc-insulin, A1B29-BisBoc-insulin and TrisBoc-insulin) were isolated and the fractions of desired (A1B29)-BisBoc-insulin were combined and lyophilized to give a white powder (72 mg).

(A1B29)-BisBoc-Insulin (51 mg) in DMF (3 mL) was reacted with MPA-OSu (36 mg) in the presence of Et_3N (30 uL). The reaction was stirred for 2 h at ambient temperature. DMF was evaporated by a under vacuum. The residue was treated with TFA (2 mL) for 10 min. and then TFA evaporated. The crude product was dissolved in water/acetonitrile (3:1) and the solution injected into a semi-preparative HPLC. Phenomenex Luna 10 m phenyl-hexyl 21 mm X 250 mm column equilibrated with an aqueous TFA solution (0.1% TFA in H_2O, solvent A) and an acetonitrile TFA solution (0.1% TFA in CH_3CN, solvent B). Elution was achieved at 9.5 mL/min by running a 27 to 32% B gradient over 120 min. Fractions containing peptides were detected by UV absorbance at 214 and 254 nm. Fractions were collected in 9.5 mL aliquots. Fractions containing the desired product profile were identified by mass detection after direct injection onto LC/MS. The pure fractions of CJC-1480 were combined and lyophilized to give a white powder (29 mg).

Mass calculated is 5958.5 g/mol, and measured by LC-MS is 5958.4 g/mol.

The amino acid sequence analysis (Edman degradation using phenylisothiocyanate) was used to confirm that the B-chain N-terminal was blocked and A-chain N-terminal (glycine) was still free.

Example III

Synthesis of (B1)-MPA-OA-insulin. (A1B29)-Boc_2-Insulin (39 mg) in DMF (3 mL) and Et3N (30 uL) was reacted with MPA-OA-OSu ([N-succinimidyl 8-N-(3-maleimidopropanylcarbonyl)aminooctanoate] 25 mg) for 4 h. DMF was evaporated and the residue treated with TFA for 10 min. After evaporation of TFA, the residue was dissolved in water/acetonitrile (1:3). The solution was injected to a semi-preparative HPLC. Phenomenex Luna 10 m phenyl-hexyl 21 mm X 250 mm column equilibrated with an aqueous TFA solution (0.1% TFA in H_2O, solvent A) and an acetonitrile TFA solution (0.1% TFA in acetonitrile, solvent B). Elution was achieved at 9.5 mL/min by running a 27 to 36% B gradient over 120 min. Fractions containing peptides were detected by UV absorbance at 214 and 254 nm. Fractions were collected in 9.5 mL aliquots. Fractions containing the desired product profile were identified by mass

detection after direct injection onto LC/MS. The pure fractions were combined and lyophilized to give a white powder (21 mg).

Mass calculated is 6099.5 g/mol, and measured by LC-MS is 6099.6 g/mol.

Example IV

Synthesis of (B29)-MPA-Insulin. Insulin (74 mg) was dissolved in DMSO (2 mL) and AcOH (46 uL). To the solution Boc_2O (6.9 mg, 2.5 equivalents) was added and the reaction was stirred for 5 h at room temperature. Water (15 mL) and acetonitrile (5 mL) were added and the solution was injected to a semi-preparative HPLC column (C18 phenyl-hexyl) in flow rate of 9.5 mL/min and with gradient from 27-40% over 120 min. The fractions at 43 min. were combined and lyophilized to give (A1B1)-Boc2-Insulin (30 mg).

(A1B1)-Boc2-Insulin (30 mg) in DMF (2 mL) and NMM (4-methylmorpnoline, 100 uL) was reacted with MPA-OSu (10 mg) for 60 min. DMF was evaporated and the residue treated with TFA for 10 min. The residue was dissolved in water/acetonitrile (3:1) and the solution injected to a semi-preparative HPLC. Phenomenex Luna 10 m phenyl-hexyl 21 mm X 250 mm column equilibrated with an aqueous TFA solution (0.1% TFA in H_2O, solvent A) and an acetonitrile TFA solution (0.1% TFA in acetonitrile, solvent B). Elution was achieved at 9.5 mL/min by running a 27 to 32% B gradient over 120 min. Fractions containing peptides were detected by UV absorbance at 214 and 254 nm. Fractions were collected in 9.5 mL aliquots. Fractions containing the desired product profile were identified by mass detection after direct injection onto LC/MS. The pure fractions were combined and lyophilized to give a white powder (22.2 mg).

Mass calculated is 5958.5 g/mol, and measured by LC-MS is 5958.0 g/mol.

The amino acid sequence analysis (Edman degradation using phenylisothiocyanate) was used to confirm that both B-chain (phenylalanine) and A-chain (glycine) N-termini were free.

***In vitro* binding assays:**

The potency of insulin derivatives was evaluated as their ability to bind the insulin receptor in human hepatocytes and to increase the glucose uptake in differentiated adipocytes.

For binding studies, HepG2 cells were used. Cells were seeded on 12-well plates at a density of 5×10^5 cells/well. Cells were used when they reached confluence. Competitive binding of unlabeled insulin and insulin derivatives was measured by overnight incubation at 4°C with ^{125}I-labeled insulin in the presence of different concentrations of unlabeled compound. All assays were performed in triplicate.

Competitive curves of insulin and insulin derivatives on intact HepG2 cells are shown on Figure 2. Values are means of three experiments done in triplicate. The resulting concentrations at 50% of inhibition (IC50) are shown in Table 1. Human insulin inhibited the binding of ^{125}I-insulin to the receptor in a dose-dependent manner with an IC50 value of 3.8×10^{-10} M. Insulin derivatives were also able to displace the binding to the insulin receptor with IC50 values of 3.7×10^{-8} M and 3.5×10^{-7} M for the derivative Example 1 and the conjugate Example 1:HSA respectively.

FIGURE 2

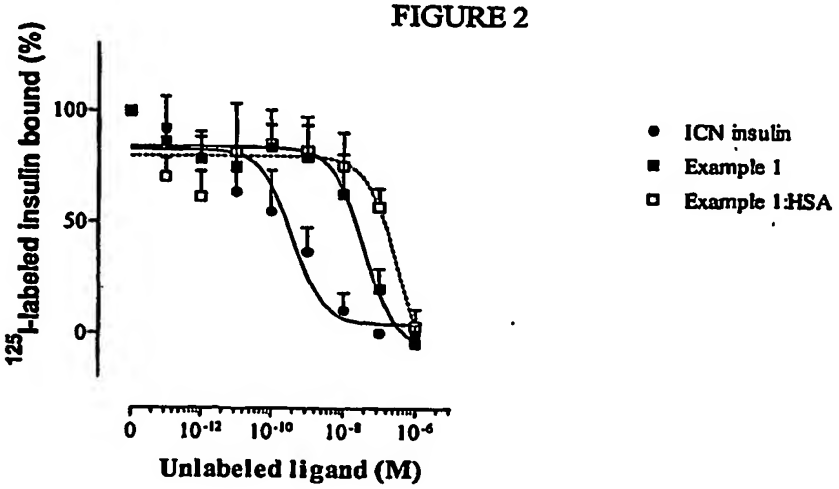


TABLE 1

	IC50 (nM)
Human Insulin	0.38
Example 1	37
Example 1:HSA	347

In vitro activity assays:

Insulin-stimulated glucose uptake was used as a measured of *in vitro* activity. 3T3-L1 preadypocytes were differentiated into adipocytes and then carried in culture. Two days after the induction of differentiation with dexamethasone, methylisobutylxanthine, and insulin, both dexamethasone, methylisobutylxanthine were removed from the culture medium. Experiments were performed with fully differentiated 3T3-L1 adypocytes between days 9 and 10. Glucose uptake assays were conducted as follow. Differentiated adipocytes were starved overnight in DMEM containing 5 mM glucose and 0.5% FBS. The cells were washed and incubated for three hours in serum starvation medium (DMEM containing 5 mM glucose and 0.2% bovine serum albumin). The cells were rinsed in Krebs-Ringer-Hepes (KRH) buffer, pH 7.5 containing 1% BSA and insulin or insulin derivative were added to the cells at different concentrations (10^{-6}-10^{-10} M) for 20 minutes at 37°C, followed by the addition of ^{14}C-2-deoxy-D-glucose (1μCi/well) for an additional 20 minutes. Non-specific uptake was evaluated in the presence of 20 μM cytochalasin B. Adipocytes were then washed three times with ice-cold KRH-5 mM glucose and solubilized in 0.1M NaOH. Radioactivity was determined in a liquid scintillation counter.

Results shown on Figures 3 and 4 demonstrate that insulin derivatives (Examples 1 and 2) and insulin derivative (Example 1: HSA) can stimulate the glucose transport in 3T3-L1 adipocytes with EC50's ranging from 2.87×10^{-9} to 4.7×10^{-12} M as compared to human insulin. The resulting concentrations at 50% of inhibition (IC50) are shown in Table 2.

FIGURE 3

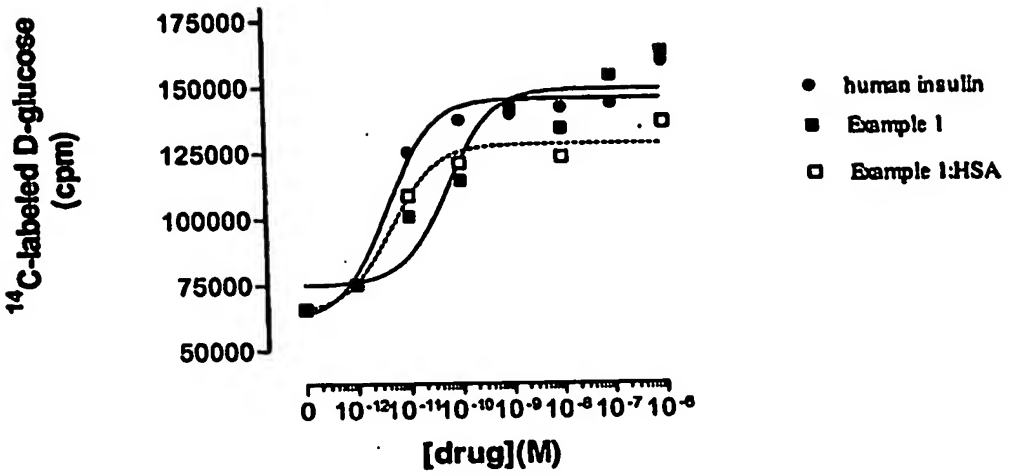


FIGURE 4

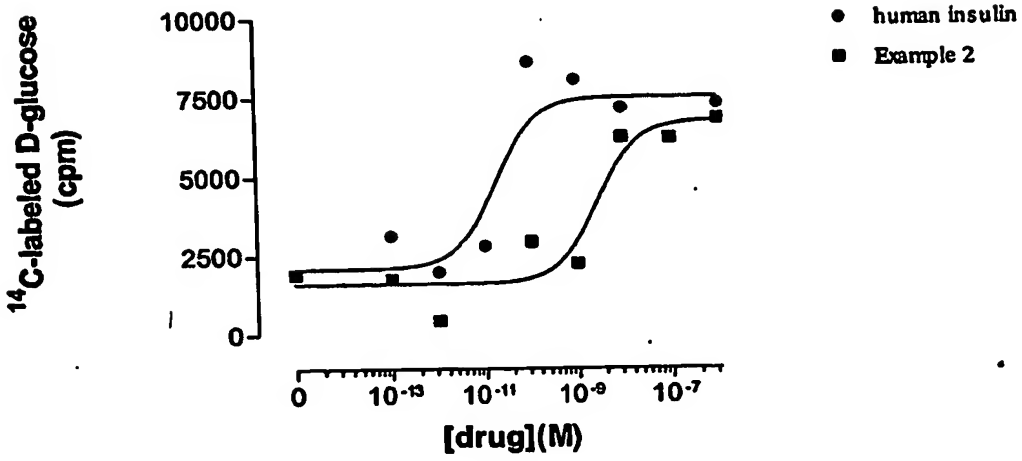


TABLE 2

	EC50
Human insulin	1.1×10^{-11}
Example 1	5.8×10^{-11}
Example 1:HSA	4.7×10^{-12}
Example 2	2.87×10^{-9}

***In vivo* experiments:**

Evaluation the blood glucose lowering efficacy of recombinant human insulin versus and insulin derivatives of the present invention when administered subcutaneously to diabetic female db/db mice, are compared.

Tested compounds were administered by a single subcutaneous bolus injection in 5-6 week-old female CD-1 mice weighing 24.3 to 33.3 g. The average volume of dosing solution injected was 0.35 mL/mouse (12.5 mL/kg).

Recombinant (E. coli) human insulin (called herein below "rH insulin") is provided by ICN™, lot # 3188F, at a concentration of 28 IU/mg.

Stock solutions of insulin derivatives were prepared at 14.29 mg/mL (~ 400 IU/mL) by reconstituting the synthesized insulin derivatives with acidified water (~ pH 2). Stock solutions were subsequently diluted with 0.9% NaCl and 0.22 µm filtered (Millex GV) to obtain the dosing solutions shown in Table 3. Group 1 received 0.9% NaCl USP as control solution.

TABLE 3

Groups	Tested Compounds	Actual Solution Concentration (mg/mL)	pH value
2	rH Insulin	0.29	3.93
3	Example 1	0.29	3.77
4	Example 1	1.43	2.88
5	Example 2	0.29	3.70
6	Example 2	1.43	2.79
7	Example 3	0.29	3.69
8	Example 4	1.43	2.98

Groups and treatments are summarized in the Table 4.

TABLE 4

Groups	Test/Control Articles	Dose Level (mg/kg)	Dose Equivalence* (~IU/kg)	Number of Animals (females)
1	0.9% NaCl	0	0	5
2	rH insulin	3.6	100	5
3	Example 1	3.6	100	5
4	Example 1	17.9	500	5
5	Example 2	3.6	100	5
6	Example 2	17.9	500	5
7	Example 3	3.6	100	5
8	Example 3	17.9	500	5

* Based on the potency of the rH insulin estimated at 28 IU/mg by the vendor.

Blood sampling (one drop) was performed *via* the tail tip and glucose levels were determined using a hand-held glucometer (Model: One Touch Ultra™, Lifescan Canada). Blood glucose levels were determined from all animals once prior to administration (pre-dose), and at 1, 2, 3, 4, 6, 24, 30, 48 and 72 hours post-dose.

***In vivo* results:**

All animals appeared normal prior to administration of the tested compounds. Approximately one hour post-dose, Group 2 of animals treated subcutaneously with 100 IU/kg of recombinant human insulin (rH insulin), exhibited slight decrease in activity and uncoordinated gait. Other treated animals appeared normal throughout the experiment. A slight decrease of food consumption was observed in animals treated with 17.9 mg /kg of compound of Example 3.

Table 5 shows food consumption (total weight/cage (g)) following a single administration of rH insulin and insulin derivatives.

TABLE 5: Food consumption: total weight/cage (g)

Groups	Treatment	24h pre-dose	0-6h	0-24h	24-48h	48-72h
1	0.9% NaCl	30.4	6.2	30.7	28.9	31.6
2	rH insulin 3.6 mg/kg	30.2	6.6	29.2	30.7	31.7
3	Example 1 3.6 mg/kg	28.7	5.8	30.5	28.8	28.7
4	Example 1 17.9 mg/kg	29.8	5.1	28.3	31.3	31.5
5	Example 2 3.6 mg/kg	31.8	6.4	30.3	29.8	31.0
6	Example 2 17.9 mg/kg	30.5	6.4	31.2	26.5	29.7
7	Example 3 3.6 mg/kg	29.9	6.0	31.4	31.9	31.8
8	Example 3 17.9 mg/kg	28.7	4.5	27.0	22.8	24.5

Table 6 shows food consumption (total weight/cage (g)) following a single administration of rH insulin and insulin derivatives.

TABLE 6: % Food consumption *versus* control group.

Groups	Treatment	24h pre-dose	0-6h	0-24h	24-48h	48-72h
1	0.9% NaCl	100.0	100.0	100.0	100.0	100.0
2	rH insulin 3.6 mg/kg	99.3	106.5	95.1	106.2	100.3
3	Example 1 3.6 mg/kg	94.4	93.5	99.3	99.7	90.8
4	Example 1 17.9 mg/kg	98.0	82.3	92.2	108.3	99.7
5	Example 2 3.6 mg/kg	104.6	103.2	98.7	103.1	98.1
6	Example 2 17.9 mg/kg	100.3	103.2	101.6	91.7	94.0
7	Example 3 3.6 mg/kg	98.4	96.8	102.3	110.4	100.6
8	Example 3 17.9 mg/kg	94.4	72.6	87.9	78.9	77.5

Delta glycaemia is calculated from blood glucose levels of post-dose glucose level versus the pre-dose glucose level for each individual mouse are reported in Figures 5, 6, 7, 8, 9, and 10. In general, insulin derivatives (Example 1, Example 2 and Example 3) were able to lower blood glucose concentrations in a dose-dependent manner and recombinant insulin, tested at one dose level only (100 IU/kg), was active for 2 hours. At 3.6 mg/kg, Example 2 was as active as insulin during the first 2 hours while only a marginal effect was observed with Example 1 and Example 3. The lowering effect of rH insulin was more pronounced at 17.9 mg/kg since it was observed for up to 24 hours. Although the overall picture tends to demonstrate that insulin derivatives were active for up to 24 hours (as compared to the control group), it is important to note that glucose levels decreased at 1-2 hours post-dose then increased at 3-4 hours and decreased again at 6 hours. This 'up and down' response might indicate that the feeding habits and the metabolism of the mice are important parameters that can affect the efficacy of the drug.

It is important to mention that db/db mice develop insulin resistance with age, which could explain the very high dose of insulin that had to be injected to observe glucose lowering effect. In a previous study with OGTT in normal mice, insulin had an effect on glucose excursion at 0.5 IU/kg.

FIGURE 5

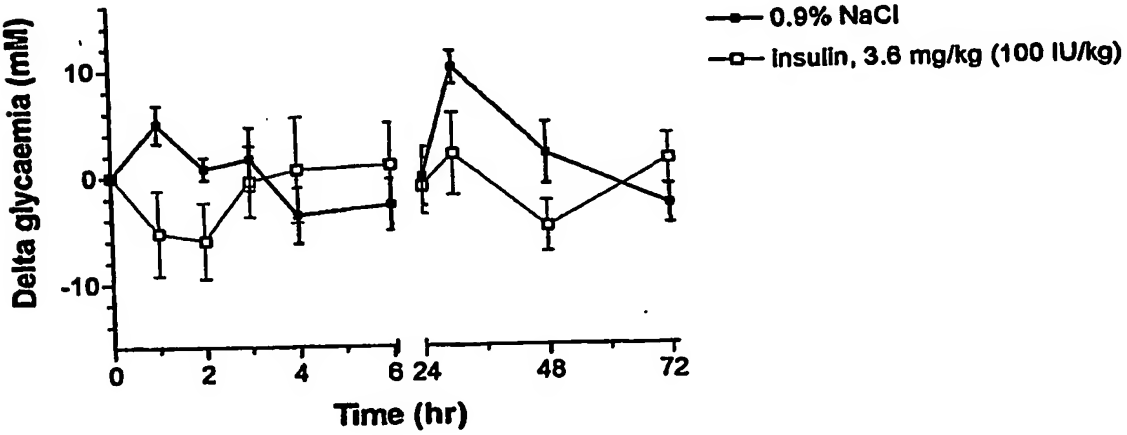


FIGURE 6

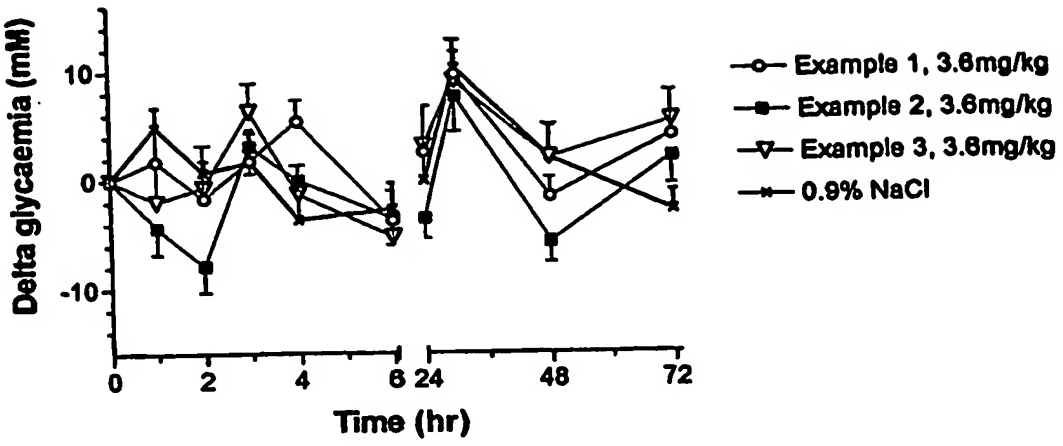

Delta glycaemia (mM)
10
0
-10
0
2
4
6
24
48
72
Time (hr)
Example 1, 3.6mg/kg
Example 2, 3.6mg/kg
Example 3, 3.6mg/kg
0.9% NaCl

FIGURE 7

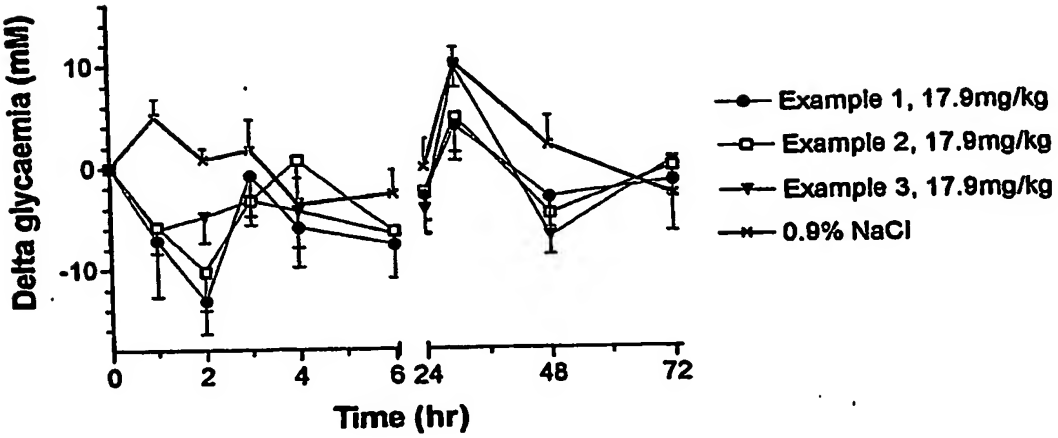

Delta glycaemia (mM)
10
0
-10
0
2
4
6
24
48
72
Time (hr)
Example 1, 17.9mg/kg
Example 2, 17.9mg/kg
Example 3, 17.9mg/kg
0.9% NaCl

FIGURE 8

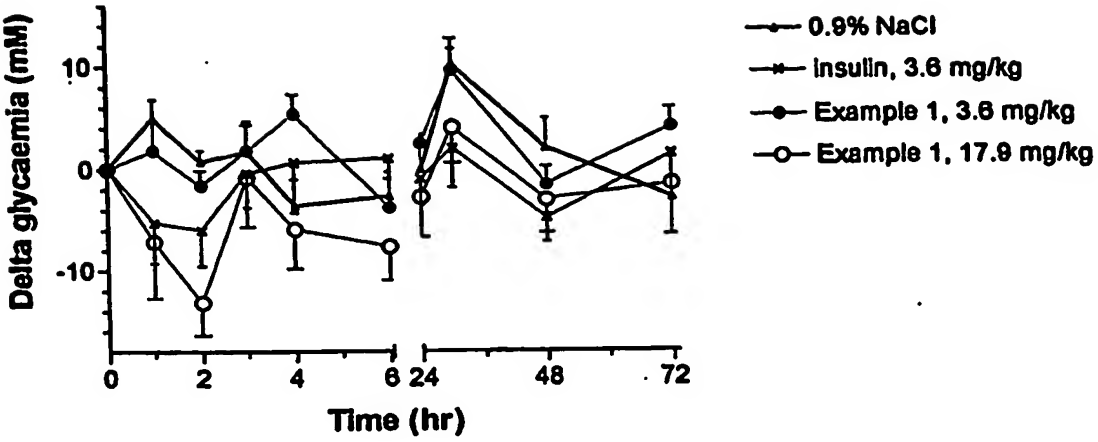


FIGURE 9

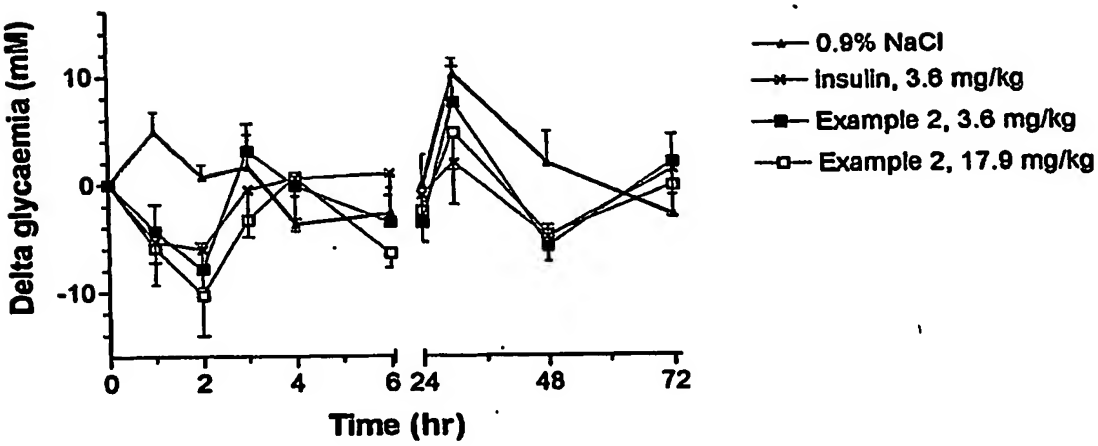
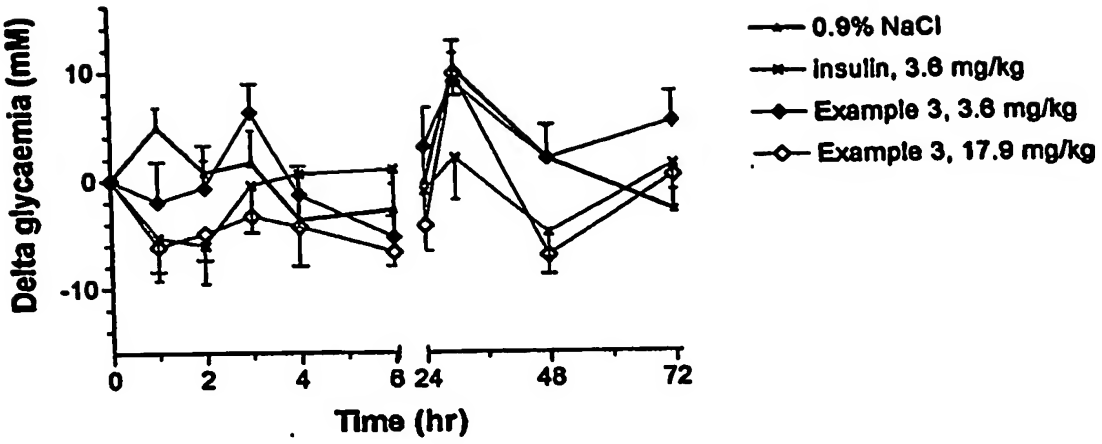


FIGURE 10



While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications, and this application is intended to cover any variations, uses or adaptations of the invention following, in general, the principles of the invention, and including such departures from the present description as come within known or customary practice within the art to which the invention pertains, and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

Applicants have not abandoned or dedicated to the public any subject matter.

The following statements of the invention are intended to characterize possible elements of the invention according to the foregoing description given in the specification. Because this application is a provisional application, these statements may become changed upon preparation and filing of the complete application. Such changes are not intended to affect the scope of equivalents according to the claims issuing from the complete application, if such changes occur. According to 35 U.S.C. § 111(b), claims are not required for a provisional application. Consequently, the statements of the invention cannot be interpreted to be claims pursuant to 35 U.S.C. § 112.

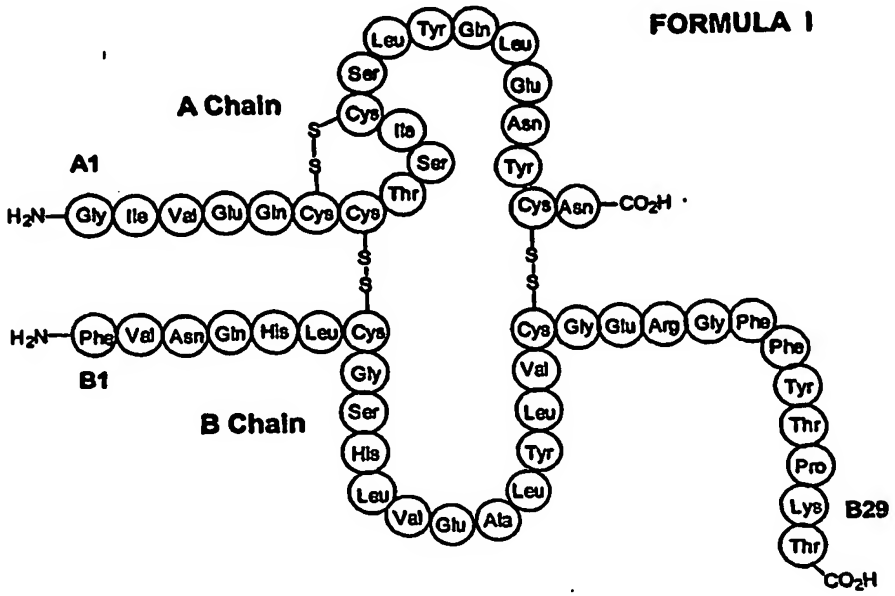
Applicants expressly do not abandon or dedicate to the public any subject matter disclosed herein.

Statements of the invention :

1. An insulin derivative comprising an insulin molecule and a reactive group capable to covalently bond a blood component *in vivo* or *ex vivo*.

2. The insulin derivative of claim 1, wherein the insulin molecule is of formula I:

FORMULA I



the reactive group being coupled to an amino acid of the insulin molecule, said amino acid being selected from the ones in positions A1, B1 and B29.

3. The insulin derivative of claim 1 or 2, wherein the reactive group selected from the group consisting of a succinimidyl-containing group and a maleimido-containing group.

4. The insulin derivative of claim 3, wherein the reactive group is a maleimido-containing group.

5. The insulin derivative of claim 4, wherein the reactive group is MPA.

6. The insulin derivative of any one of claims 1 to 5, wherein the reactive group is coupled to an amino acid of the insulin molecule via a linker; the linker being selected

from the group consisting of AEEA, AEEA-AEEA, EDA and -NH_2-$(CH_2)_n$-COOH where n is an integer between 1 and 20.

7. The insulin derivative of claim 6, wherein the linker is -NH_2-$(CH_2)_7$-COOH.

8. An insulin conjugate comprising an insulin derivative according to any one of claims 1 to 7, wherein the reactive group has reacted with a blood component *in vivo* or *ex vivo* so as to form a covalent bond.

9. The insulin conjugate of claim 8, wherein the blood component is a blood protein.

10. The insulin conjugate of claim 9, wherein the blood protein is serum albumin.

11. A method for treating a glycaemic-related disease or disorder, comprising the administration of the insulin derivative according to any one of claims 1 to 7.

12. A method according to claim 11, wherein the glycaemic-related disease is diabetes of type I or II.

13. A method for treating a glycaemic-related disease or disorder, comprising the administration of the insulin conjugate according to any one of claims 8 to 10, where the covalent bond was formed *ex vivo*.

14. A method according to claim 13, wherein the glycaemic-related disease is diabetes of type I or II.

15. Use of the conjugate defined in any one of claims 8 to 10, for the preparation of a medicament for the treatment of a glycaemic-related disease or disorder.

16. Use according to claim 15, wherein the glycaemic-related disease is diabetes of type I or II.